Staphylococcus aureus sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections

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Many Gram-positive bacteria covalently tether their surface adhesins to the cell wall peptidoglycan. We find that surface proteins of *Staphylococcus aureus* are linked to the cell wall by sortase, an enzyme that cleaves polypeptides at a conserved LPXTG motif. *S. aureus* mutants lacking sortase fail to process and display surface proteins and are defective in the establishment of infections. Thus, the cell wall envelope of Gram-positive bacteria represents a surface organelle responsible for interactions with the host environment during the pathogenesis of bacterial infections.

Gram-positive bacteria are surrounded by a cell wall envelope containing attached polypeptides and polysaccharides (1). Although surface proteins of Gram-positive bacteria have long been characterized as adhesins for human tissues, the mechanism of their cell wall attachment and surface display has only recently been described. Protein A, a surface protein of *Staphylococcus aureus* (2), is synthesized as a precursor bearing an N-terminal signal peptide and a C-terminal sorting signal with an LPXTG motif (3). After signal peptide-mediated initiation of the precursor into the secretory pathway, the sorting signal directs protein A to the cell wall envelope (4). The polypeptide is then cleaved between the threonine and the glycine of the LPXTG motif (5). The liberated carboxyl group of threonine forms an amide bond with the amino group of the pentaglycine crossbridge (6), thereby tethering the C terminus of protein A to the bacterial peptidoglycan (7, 8).

To identify genes that act in the sorting pathway of protein A, temperature-sensitive staphylococcal mutants were pulse-labeled and screened for a defect in protein A precursor processing (9). A mutant *S. aureus* strain was identified, transformed with a plasmid library of staphylococcal genomic DNA, and screened for complementation. The *srtA* gene (surface protein sorting A) restored the defect in cell wall anchoring of protein A (9). Furthermore, purified SrtA catalyzed the *in vitro* cleavage of peptides bearing the LPXTG motif (10). These results suggest that *srtA* encodes sortase, a transpeptidase responsible for processing the sorting signal of protein A.

The genome of *S. aureus* encodes at least 10 different surface proteins bearing C-terminal sorting signals with an LPXTG motif (11). Many of these polypeptides are known to interact with various human tissues, serum proteins, or polypeptides of the extracellular matrix (12). For example, protein A (Spa) binds to the Fc portion of immunoglobulins (13), a mechanism that is thought to prevent opsonophagocytosis of staphylococci after their entry into the human host (14, 15). Binding of the clumping factors, ClfA and ClfB, to fibrinogen promotes bacterial adhesion to vascular and endocardic lesions (16–18). The FnbA and FnbB surface proteins bind to fibronectin (19, 20). This interaction allows staphylococci to adhere to various tissues and, similar to fibronectin-binding proteins of *Streptococcus pyogenes* (21), presumably provides for the invasion and apoptotic death of infected epithelial cells (22). Although all of these surface proteins are thought to be essential for the pathogenesis of staphylococcal infections (12), this assumption has not been demonstrated for surface protein knockout strains in animal models of infection (15, 23). Presumably, surface proteins of *S. aureus* fulfill at least partially redundant functions.

Previous work left unresolved whether *srtA* is absolutely required for the anchoring of surface proteins to the cell wall envelope. We find that *S. aureus* mutants lacking the *srtA* gene fail to anchor all surface proteins examined because of a defect in the processing of sorting signals at the LPXTG motif. As a result, the assembly and display of surface adhesins is abolished, causing a reduction in the ability of sortase mutants to establish animal infections.

Experimental Procedures

Bacterial Strains and Plasmids. *S. aureus* strains RN4220 (*res*$^-$) (24), OS2 (*spa*$^-$*:ermC*) (3), and Newman (25) have been described. *srtA* sequences were PCR amplified with the primers GSA1–4 (AAGGATCCAAAAGGAGCGGTATACATTGC) and orf6-3K (AAAGGTACCGTGTACTTTAAAGTTGGTAATG) as well as orf6-5K (AAAGGTACCCTTTTTATCTTTACTCGCC) and orf6-3E (AAAGAATTCGAACCACTACATAATAAATC). The DNA fragments were digested with *Bam*HI and *Kpn*I or *Eco*RI and *Kpn*I, ligated, and inserted between the *Eco*RI and *Bam*HI sites of pTS1 (26) to generate pSM29. *ermC* was PCR amplified with the primers ErmC-5′ (AAAGGTACCTACACCTCCGGATAATAAA) and ErmC-3′ (AAAGGTACCCACAAGACACTCTTTTTTC). The *Kpn*I-digested DNA fragment was inserted into pSM29 to yield pSrtA-KO. RN4220 (pSrtA-KO) was plated at 42°C on erythromycin plates (10 μg/ml). Colonies were picked and analyzed by PCR using the primer pairs GSA1–4/orf6-3E and ErmC-5′/ErmC-3′. Plasmids pSeb-Spa$_{KpnI}$ and pSeb-FnbA have been described previously (4). pSeb-ClfA was constructed by PCR amplification of a DNA segment specifying the clumping factor sorting signal. The PCR product was digested with *Kpn*I and *Bam*HI and inserted into pSeb-Spa$_{KpnI}$ cut with the same restriction enzymes. Plasmids pSeb$_{SP}$-BlaZ (27), pSeb-Cws-BlaZ, and pSeb-Cws$_{\Delta LPXTG}$-BlaZ (5) have been previously constructed. To generate pSeb-Cws$_{\Delta R}$-BlaZ, the Spa sorting signal was PCR amplified with the primers Seb-5 (AAGGTACCTTTCTTTGTCGTAAGATAAACTTCA) and T-Sac (AAGAGCTCCCAGCTAATGCTGCACCT), cut with *Kpn*I–*Sac*I, and inserted into pSeb-Cws-BlaZ cut with the same restriction

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Abbreviations: Clf, clumping factor; Fnb, fibronectin-binding protein; Seb, staphylococcal enterotoxin B; Spa, staphylococcal protein A; SrtA, staphylococcal surface protein sorting A; TCA, trichloroacetic acid; cfu, colony-forming unit.

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enzymes. Pulse–chase, cell fractionation, and cell wall linkage assay were performed as previously described (4).

Immunofluorescence Microscopy. Staphylococci were grown in TSB, washed with PBS, and applied to poly-L-lysine-coated glass slides. Slides were washed with PBS, blocked for 1 h with 2% BSA in PBS, and incubated with a 1:1,000 dilution of CY3-labeled IgG in PBS/BSA for 1 h. Slides were washed with PBS and viewed under fluorescence excitation, and images were captured with a charge-coupled device camera.

Animal Experiments. Staphylococci were grown overnight in tryptic soy broth (TSB), diluted into fresh medium, grown for 3 h at 37°C to OD_{600} 0.5, and washed and diluted in PBS. Six- to eight-week-old C57BL/6 mice or Swiss–Webster mice were inoculated with 500 μl of staphylococcal suspension into the tail vein. Five days after infection, mice were euthanized with CO_2. Kidneys were excised, weighed, and homogenized in 0.5% Triton X-100. Staphylococci were counted by dilution and colony formation. All experiments used staphylococcal strains that were subjected to animal passage and isolated from the kidneys of infected mice. All mice were treated in accordance with the institutional guidelines for the humane care and treatment of animals.

Results

Staphylococcal Sortase. To generate knockout mutations of sortase, the *ermC* gene, flanked by *srtA* nucleotide sequences, was cloned into an *Escherichia coli–S. aureus* shuttle plasmid that is temperature-sensitive for replication (26). Homologous recombination caused the insertion of *ermC* into the genome of *S. aureus* RN4220, replacing codons 52–142 of *srtA* with *ermC*. To facilitate virulence studies, the *srtA:ermC* allele was transduced with phage Φ85 into *S. aureus* Newman, a strain previously isolated from a human infection (25). Transductants were selected on erythromycin plates and analyzed by PCR amplification with primers that anneal to *ermC* or to sequences flanking the *srtA* gene (Fig. 1*A*). The *ermC* gene could be amplified from the chromosomal DNA of the *srtA*$^-$ mutant strains SKM1 and SKM3 but not from the isogenic parent strains RN4220 and Newman. Amplification with primers specific for sequences flanking *srtA* revealed the insertion of *ermC* into the *srtA* genes of strains SKM1 and SKM3.

Recombinant sortase lacking the N-terminal signal peptide ($SrtA_{\Delta N}$) was purified from the cytoplasm of *E. coli* and used to raise specific antibodies (10). Immunoblotting of staphylococcal extracts with α-SrtA revealed that sortase is a polypeptide of 26 kDa (Fig. 1*B*). Wild-type sortase migrated more slowly on SDS/PAGE than $SrtA_{\Delta N}$, suggesting that the N-terminal signal peptide of sortase was not cleaved. The *srtA*$^-$ strains SKM1 and SKM3 did not express sortase, however, transformation of the mutant staphylococci with plasmid-encoded wild-type *srtA* restored expression. To localize sortase within staphylococci, *S. aureus* RN4220 cultures were fractionated into the extracellular medium (MD), cell wall digest (CW), cytosol (C), and membrane compartments (M) (Fig. 1*C*). Sortase was found only in the membrane, suggesting that the N-terminal signal peptide functions also as a membrane anchor.

Anchoring Surface Proteins to the Cell Wall of *S. aureus*. To test whether sortase mutants are defective in the anchoring of staphylococcal surface proteins, we investigated the processing of C-terminal sorting signals by pulse-labeling staphylococcal cultures. Sorting signals of protein A (Spa), fibronectin-binding protein (FnbA), and clumping factor (ClfA) were fused to the C terminus of the normally secreted enterotoxin B reporter protein. Wild-type staphylococci exported surface protein precursor (P1) from the cytoplasm and removed the N-terminal signal peptide to generate the P2 intermediate. P2 was cleaved by sortase at the LPXTG motif to generate mature, cell wall-anchored surface protein (M) (Fig. 2). Wild-type staphylococci cleaved the Spa, FnbA, and ClfA sorting signals to generate mature surface protein (Fig. 2*B*). In contrast, the sortase mutant strains SKM1 and SKM3 (*srtA*$^-$) failed to cleave all P2 surface protein precursors. Transformation of sortase mutant strains with plasmids encoding wild-type *srtA* restored the processing of C-terminal sorting signals.

As sortase mutant staphylococci cannot process C-terminal sorting signals, these strains are likely defective in anchoring surface proteins to the cell wall envelope. The subcellular location of surface proteins was examined by fractionating pulse-labeled staphylococci into the medium, cell wall, cytoplasm, and membrane compartments. Wild-type staphylococci anchored surface proteins to the cell wall envelope. In contrast, sortase mutant staphylococci mislocalized surface proteins, and the P2 precursors were immunoprecipitated from the cytoplasm, membrane, and cell wall compartment (Fig. 2*C*). This phenotype is identical to the missorting of protein A mutants lacking the LPXTG motif. As the LPXTG mutant is not stably inserted into

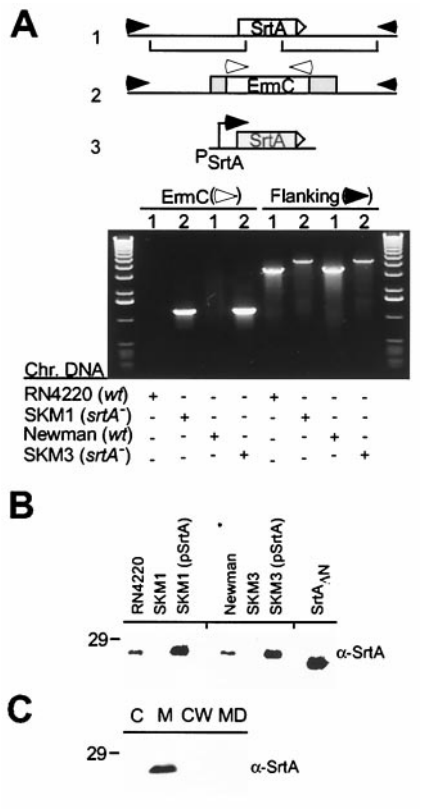

Fig. 1. *S. aureus* sortase (*srtA*) mutants. (*A*) Drawing depicts the wild-type sortase (*srtA*) gene (1) and the *srtA*$^-$*:ermC* allele (2). Plasmid-encoded wild-type *srtA* (pSrtA) is expressed from its own promoter and was used for complementation studies (3). Oligonucleotide primers binding to sequences flanking the *srtA* gene (filled arrows) or the *ermC* gene (open arrows) were used to amplify DNA fragments from the chromosomal DNA of strains RN4220 (*srtA*, 1), SKM1 (*srtA*$^-$, 2), Newman (*srtA*, 1), and SKM3 (*srtA*$^-$, 2). DNA fragments were separated on ethidium bromide-stained agarose gel, flanked by the 1-kb DNA ladder. (*B*) Immunoblotting with anti-SrtA (α-SrtA) revealed the presence of sortase (26 kDa) in extracts of wild-type strains RN4220 and Newman and the absence of sortase in the mutant strains SKM1 and SKM3 (*srtA*$^-$). $SrtA_{\Delta N}$ lacking the N-terminal membrane anchor was expressed in *E. coli* and purified. (*C*) RN4220 cultures were fractionated into medium (MD), cell wall (CW), membrane (M), and cytosolic (C) compartments and immunoblotted with α-SrtA.

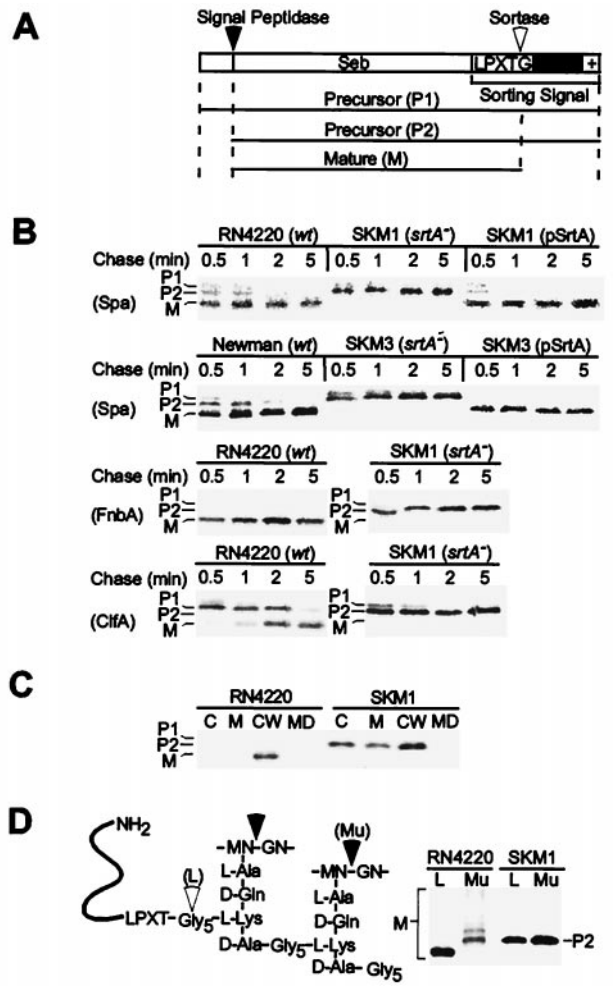

Fig. 2. Anchoring surface proteins to the cell wall of staphylococci. (*A*) Drawing depicts the structure of the Seb-Cws surface protein, which is comprised of enterotoxin B (Seb) with an N-terminal signal peptide and a C-terminal cell wall sorting signal. The cell wall sorting signal contains an LPXTG motif, a hydrophobic domain (black box), and a positively charged tail. (*B*) The sorting signals of protein A (Spa), fibronectin-binding protein (FnbA), and clumping factor (ClfA) were fused to Seb, and cell wall sorting was followed in a pulse–chase experiment. (*C*) Pulse-labeled staphylococci cultures were fractionated into medium (MD), cell wall (CW), membrane (M), and cytosolic (C) compartments, and Seb-Spa$_{490-524}$ was immunoprecipitated with anti-Seb (α-Seb). (*D*) The peptidoglycan of staphylococci was digested with lysostaphin (L) or mutanolysin (Mu), and radiolabeled surface proteins were analyzed by SDS/PAGE.

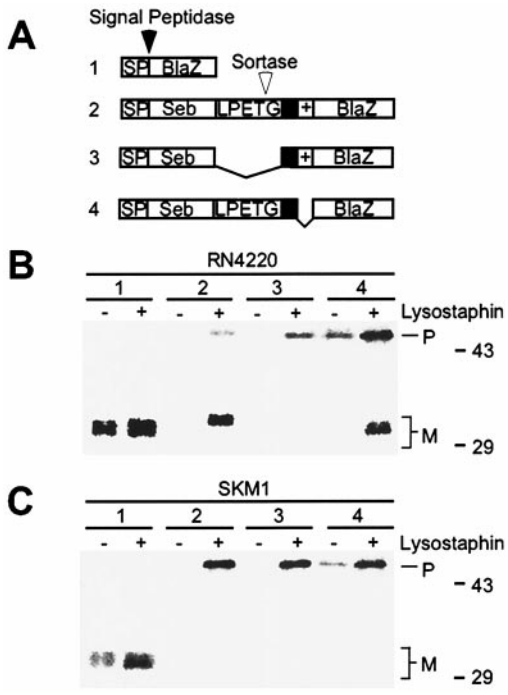

Fig. 3. Protein secretion and sorting pathways of staphylococci. (*A*) Drawing depicts the structure of protein fusions with the mature domain of staphylococcal β-lactamase (BlaZ). 1, (Seb$_{SP}$-BlaZ) fusion of the enterotoxin B signal peptide (SP). 2, (Seb-Cws-BlaZ) fusion of enterotoxin B (Seb) and the protein A sorting signal to BlaZ. 3, (Seb-Cws$_{\Delta LPXTG}$-BlaZ) same fusion as in 2 but lacking the LPXTG motif. 4, (Seb-Cws$_{\Delta R}$-BlaZ) same fusion as in 2 but lacking the retention signal (+). (*B*) Pulse-labeled staphylococcal cultures (strain RN4220) were divided into two aliquots and precipitated with TCA. One sample was directly boiled in SDS, whereas the other was first subjected to peptidoglycan hydrolysis with lysostaphin and then boiled in SDS. Samples were subjected to immunoprecipitation with anti-BlaZ (α-BlaZ) and analyzed by SDS/PAGE and PhosphorImager. (*C*) Same experiment as in *B*, but using the sortase mutant strain SKM1.

the cytoplasmic membrane, the polypeptide appears during fractionation in several different compartments (3). To measure the covalent linkage of surface proteins to the cell wall, the staphylococcal peptidoglycan was digested with two different enzymes (Fig. 2*D*). Lysostaphin (L) cut at the pentaglycine crossbridge (28) and released cell wall-anchored surface protein with uniform migration on SDS/PAGE. Mutanolysin (Mu) cleaves the glycan strands (29), solubilizing surface protein as a spectrum of fragments with linked peptidoglycan. Mutanolysin digestion of the peptidoglycan of strain SKM1 released unlinked P2 precursor, demonstrating the inability of sortase mutants to anchor surface proteins to the cell wall.

Staphylococcal Protein Secretion and Sorting Pathways. Precursor proteins bearing N-terminal signal peptides are directed to the secretion machinery of Gram-positive bacteria, translocated across the cytoplasmic membrane, and secreted into the extracellular environment. Surface proteins of *S. aureus* are initiated into the secretion pathway, where the C-terminal sorting signals function to retain surface proteins within the secretion machinery, allowing processing at the LPXTG motif. We explored whether sortase mutants were defective at a distinct step of cell wall anchoring or caused a general disruption of the protein secretion and sorting pathways. The staphylococcal cell wall is a covalently linked network that is impermeable to proteins (1). When pulse-labeled cultures are precipitated with trichloroacetic acid (TCA) and boiled in SDS, only secreted proteins are solubilized. In contrast, staphylococcal surface proteins, membrane, or cytoplasmic proteins require digestion of the cell wall with lysostaphin for solubilization in hot SDS.

Fusion of the N-terminal signal peptide of enterotoxin B (Seb) (30) to the mature domain of staphylococcal β-lactamase (BlaZ) generated a hybrid protein (Seb$_{SP}$-BlaZ, 1) that is initiated into the secretory pathway and translocated into the extracellular medium by wild-type staphylococci (27) (Fig. 3). The sorting signal of protein A (Cws) was placed into the center of a hybrid polypeptide, flanked at the N terminus by Seb and at the C terminus by BlaZ (Seb-Cws-BlaZ, 2) (5). Wild-type staphylococci cleaved the Seb-Cws-BlaZ (2) precursor (P) at the LPXTG motif and generated the mature C-terminal BlaZ fragment (M). Both precursor and mature species were not secreted into the medium. Removal of the LPXTG motif abolished precursor cleavage of the hybrid protein (Seb-Cws$_{\Delta LPXTG}$-BlaZ, 3),

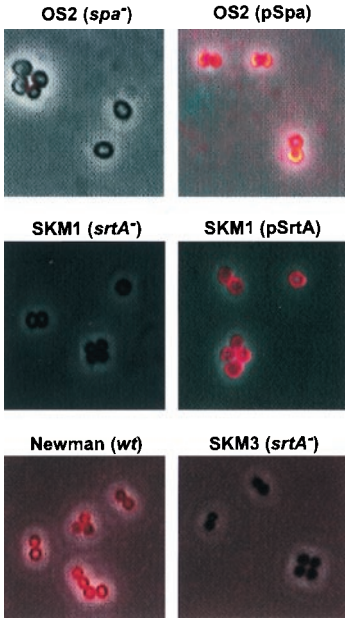

Fig. 4. Display of protein A on the staphylococcal surface. Binding of CY3-labeled Ig to protein A was measured by capturing dark-field and fluorescent microscopy images with a charge-coupled device camera and superimposing the data. *S. aureus* OS2 (*spa*$^-$) cannot express protein A; however, Ig binding was restored by transformation with plasmid encoding wild-type *spa* (pSpa). *S. aureus* SKM1 (*srtA:ermC*) failed to bind CY3-labeled Ig, a defect that was complemented by transformation with plasmids encoding wild-type *srtA* (pSrtA). *S. aureus* Newman (wild-type, *wt*) displayed protein A on the staphylococcal surface; however, the isogenic sortase knockout mutant SKM3 failed to bind CY3-labeled Ig.

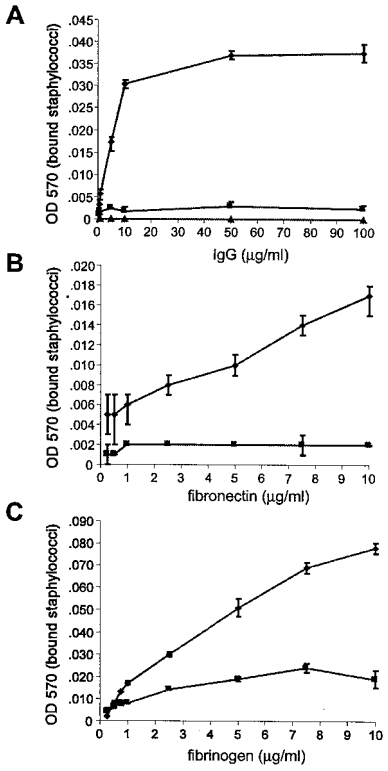

Fig. 5. Display of surface proteins by staphylococci. Microtiter dishes were coated with increasing amounts of Ig, fibronectin, or fibrinogen. Binding of staphylococci to mammalian proteins was detected by staining with crystal violet and measuring the absorbance at 570 nm in a spectrophotometer. (*A*) Binding of *S. aureus* RN4220 (◆), OS2 (*spa*$^-$, ▲), and SKM1 (*srtA*$^-$, ■) to Ig. (*B*) Binding of *S. aureus* RN4220 (◆), and SKM1 (*srtA*$^-$, ■) to fibronectin. (*C*) Binding of *S. aureus* Newman (◆) and SKM3 (*srtA*$^-$, ■) to fibrinogen.

whereas removal of the positively charged retention signal caused the secretion of uncleaved precursor molecules into the extracellular medium (Seb-Cws$_{\Delta R}$-BlaZ, 4). Sortase mutant cells did not display a defect in the staphylococcal secretion pathway as Seb$_{SP}$-BlaZ and Seb-Cws$_{\Delta R}$-BlaZ were found in the extracellular medium. Sortase mutants failed to cleave Seb-Cws-BlaZ at the LPXTG motif. Nevertheless, the hybrid protein required lysostaphin digestion of the cell wall for solubility in hot SDS, indicating that the polypeptide was retained within the secretory pathway. Thus, sortase mutants are defective in the cleavage and anchoring of surface proteins, but unaffected in the protein secretion pathway.

Display of Surface Proteins by Staphylococci. We asked whether sortase mutant strains were able to assemble and display surface adhesins. Assembly of functional adhesins was examined by incubation of staphylococci with specific ligands, i.e., mammalian plasma and extracellular matrix proteins. CY3-labeled Ig was added to staphylococci, and binding to protein A on the bacterial surface was visualized by fluorescence microscopy (Fig. 4). *S. aureus* Newman (wild-type *spa*) bound CY3-labeled Ig on the cell surface as revealed by the halo of fluorescence surrounding bacteria. In contrast, the sortase mutants SKM1 and SKM3 (*srtA*$^-$) failed to bind CY3-labeled Ig. Binding was restored to wild-type levels by transformation of the mutant strains with plasmids encoding wild-type sortase (pSrtA). As a control, *S. aureus* OS2 lacking the protein A gene (*spa*$^-$) also did not bind Ig, a defect that was corrected by the introduction of plasmids specifying the wild-type *spa* gene.

We wondered whether the functional assembly of all staphylococcal adhesins, protein A, fibronectin-binding proteins (FnbA and FnbB), and clumping factors (ClfA and ClfB) may be affected in the sortase mutant strains. Purified Ig, fibronectin, or fibrinogen was immobilized on microtiter plates. Binding of staphylococci to mammalian proteins was observed by staining bacteria with crystal violet and measuring the absorbance at 570 nm in a spectrophotometer (Fig. 5) (31). The addition of increasing amounts of Ig to microtiter dishes caused increasing numbers of *S. aureus* RN4220 to bind to the coated plates. Sortase mutant staphylococci (SKM1) failed to bind Ig, consistent with the previous observation that the display of Spa on the bacterial surface was indeed abolished. As a control, the Spa-deficient strain OS2 did not bind to Ig-coated microtiter plates. The addition of increasing amounts of fibronectin to microtiter dishes caused increasing numbers of *S. aureus* RN4220 to bind to the coated plates. The sortase mutation abolished all fibronectin binding of strain SKM1. During exponential growth, *S. aureus* Newman is known to display ClfA and ClfB, each of which promotes binding to fibrinogen (17). In contrast to *S. aureus* Newman, the isogenic sortase mutant, strain SKM3, failed to bind fibrinogen. Thus, sortase is absolutely necessary for the assembly and display of functional surface adhesins in the cell wall envelope of staphylococci.

Surface Proteins and the Pathogenesis of *S. aureus* Infections. *S. aureus* is a resident of the human skin and nares. What distinguishes *S. aureus* strains from other microbes is their ability to penetrate deeper layers of the skin, thereby causing suppurative diseases or purulent wound infections. Following entry into the blood stream, *S. aureus* escape phagocytic killing by immune cells and, after binding to specific tissues, cause abscesses in internal organs (32). To test whether surface adhesins

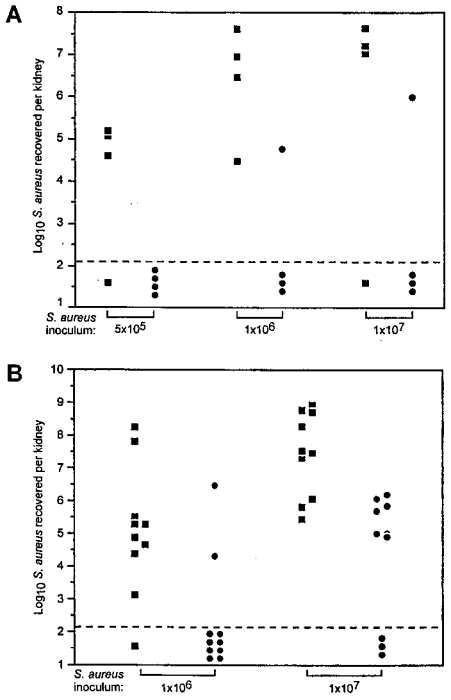

Fig. 6. Surface proteins and the pathogenesis of *S. aureus* infections. *S. aureus* Newman (human clinical isolate) and the isogenic sortase mutant SKM3 were injected into the tail vein of C57BL/6 (*A*) or Swiss–Webster mice (*B*) as indicated. Five days after infection, animals were killed, kidneys excised, homogenized, and plated. Symbols indicate cfu of *S. aureus* Newman (■) and the sortase mutant strain SKM3 (●). The dashed line represents the limit of detection of staphylococci in renal tissues. *P* values were calculated after log transformation of the data using the Student *t* test or the Mann–Whitney *U* test (parenthesis): C57BL/6: 5×10^5 cfu, $P = 0.03$ (0.08); 1×10^6 cfu, $P = 0.01$ (0.04); 1×10^7 cfu, $P = 0.12$ (0.11). Swiss–Webster: 1×10^6 cfu, $P = 0.049$ (0.006); 1×10^7 cfu, $P = 0.0004$ (0.001).

are necessary for the pathogenesis of staphylococcal diseases, we investigated the formation of renal abscesses in a mouse model of infection (33). After injection into the tail vein of animals, staphylococci escape phagocytic killing and infect kidney tissues and produce renal abscesses after an interval of 5 days (34). Quantification of viable bacteria within kidneys is a measure for staphylococcal colonization and multiplication within tissues of the infected host.

C57BL/6 inbred mice were injected with 5×10^5, 1×10^6, or 1×10^7 colony-forming units (cfu) of *S. aureus* Newman. Almost all infected animals developed kidney abscesses (Fig. 6). The number of viable staphylococci within these abscesses varied between 5×10^4 and 8×10^7 cfu. This variation is likely because of differences in the innate immune response between animals. Injection of C57BL/6 mice with 5×10^5, 1×10^6, or 1×10^7 cfu of the sortase mutant strain SKM3 revealed a dramatic reduction in virulence. Almost all animals cleared the inoculum. Only two animals developed abscesses with viable staphylococci (8×10^4 and 1×10^6 cfu, respectively). Similar results were observed with outbred Swiss–Webster mice. On injection of 1×10^6 cfu, *S. aureus* Newman caused kidney abscesses in almost all infected animals. These lesions contained variable numbers of staphylococci (2×10^3 to 1×10^7 cfu), reflecting the inherent heterogeneity of the immune system of outbred mouse strains. Nevertheless, almost all mice injected with 1×10^6 cfu of strain SKM3 cleared the inoculum. Kidney abscesses of the sortase mutant strain SKM3 could be observed on injection of 1×10^7 cfu. These abscesses contained on average 1/100 as many staphylococci as similar lesions caused by infection with *S. aureus* Newman.

Table 1. Staphylococcal surface proteins are required to establish acute infections in mice

S. aureus strain	cfu injected	Mortality, %
	Experiment 1	
Newman (wt)	1.25×10^8	90
Newman (wt)	1.25×10^7	90
Newman (wt)	1.25×10^6	0
SKM3 (*srtA*$^-$)	1.35×10^8	20
SKM3 (*srtA*$^-$)	1.35×10^7	0
SKM3 (*srtA*$^-$)	1.35×10^6	0
	Experiment 2	
Newman (wt)	7.0×10^7	100
Newman (wt)	7.0×10^6	40
Newman (wt)	7.0×10^5	0
SKM3 (*srtA*$^-$)	1.1×10^8	40
SKM3 (*srtA*$^-$)	1.1×10^7	0
SKM3 (*srtA*$^-$)	1.1×10^6	0

S. aureus strains Newman (wild-type) and the isogenic sortase mutant SKM3 were grown overnight in TSB supplemented with 5% sheep's blood at 37°C. The cultures were diluted 1:10 in fresh medium and incubated with shaking for 5 h. Staphylococci were diluted into 5% hog gastric mucin, and 0.5 ml was injected i.p. into 10 CD1 mice per dilution. Animals were observed for 72 h, and moribund mice were euthanized. Average LD_{50} Newman 7.2×10^6 (Exp. 1), 7.58×10^6 (Exp. 2) and SKM3 1.8×10^8 (Exp. 1), 1.2×10^8 (Exp. 2) ($P < 0.05$).

Because of their ability to elicit an effective immune response, infected mice can eventually clear almost all *S. aureus* infections. Hence, after injection into the peritoneal cavity, large numbers of staphylococci are required to cause a lethal infection (35). Lethal infections are thought to measure the sum of all virulence factors that bacterial pathogens elaborate (36). For example, in addition to the display of surface adhesins, *S. aureus* secrete numerous exotoxins and superantigens to manipulate the immune system and escape phagocytosis. To evaluate the contribution of surface proteins during the pathogenesis of acute infections, staphylococci were injected into the peritoneal cavity of CD1 mice (Table 1). In two independent experiments, wild-type *S. aureus* Newman caused a lethal infection in half of all infected animals when 8.09×10^6 cfu were injected into the peritoneal cavity of CD1 mice. In contrast, the isogenic sortase mutant strain SKM3 required 2.15×10^8 cfu to produce a lethal disease in half of the infected animals. These data indicate that the sortase mutant strain is significantly impaired in the ability to produce an acute infection.

Discussion

S. aureus sortase is a membrane-anchored enzyme and is absolutely required for the anchoring of all surface proteins to the cell wall envelope. Biochemical studies revealed that sortase cleaves surface protein precursors between the threonine and the glycine of the LPXTG motif (5, 10). Anchoring occurs by a transpeptidation reaction, and sortase captures the C-terminal carboxyl of cleaved surface proteins by the formation of a thioester bond with its active site sulfhydryl (10). Nucleophilic attack of the amino group of the lipid II peptidoglycan precursor presumably completes the sorting reaction, causing surface proteins to form an amide bond with the cell wall crossbridge and regenerating the active site sulfhydryl of sortase (37). Sortase utilizes precursor molecules of both the protein secretion and cell wall synthesis pathways as substrates for the transpeptidation reaction (38). Determination of the subcellular distribution of sortase in the cytoplasmic membrane may reveal how the enzyme can gain access to these molecules.

Sortase likely plays a universal role in Gram-positive bacteria. Surface proteins harboring C-terminal sorting signals with an LPXTG motif have been found in all pathogenic Gram-positive bacteria (1). Further, the amino group of the cell wall crossbridge is a conserved feature of all of these microorganisms (39). Sortase homologs have been identified in *Actinomyces naeslundi*, *Bacillus subtilis*, *Clostridium perfringens*, *Corynebacterium diphtheriae*, *Enterococcus faecalis*, *Streptococcus mutans*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* (9). These homologs may have a function similar to *S. aureus* sortase. For example, *Actinomyces naeslundi* elaborate fimbriae that are composed of subunits bearing C-terminal sorting signals with an LPXTG motif (40). Fimbrial assembly is reported to depend on the actinomycetal sortase homolog (41). Furthermore, characterization of the anchor structure of surface proteins in *Listeria monocytogenes* revealed that cell wall sorting occurs by a mechanism similar to that described in staphylococci (42).

We demonstrate here that sortase is essential for the functional assembly of all surface proteins examined and for the pathogenesis of *S. aureus* infections. Compounds that interfere with sortase function may be useful for the treatment of human infections caused by Gram-positive bacteria. Sortase inhibitors should act as antiinfective agents and disrupt the pathogenesis of bacterial infections without affecting microbial viability. Antiinfectives may not exert selective pressure toward the development of bacterial resistance. In contrast, antibiotics, which aim to kill all microbes, do exert strong selective pressure, resulting in the emergence of drug-resistant strains (43). Because of the massive consumption of antibiotics, drug-resistant microbes already predominate as human pathogens (44). Modern chemotherapy must consider the threat of antibiotic resistance and target factors that are required for the pathogenesis of bacterial infections.

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